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MECHANISMS OF ACTION OF ESE1, A NOVEL TRANSCRIPTIONAL REGULATOR OF CARTILAGE REMODELING, IN MMP-13 REGULATION

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Purpose: Matrix metalloproteinase (MMP)-13 plays a major role in cartilage degradation. MMP-13 expression and activity are up-regulated in osteoarthritis (OA) cartilage as compared to non-OA cartilage. We previously reported that *ELF3/ESE1* levels are higher in OA cartilage and that ESE-1 transactivates the *MMP13* promoter via a proximal highly conserved ETS binding site. The aim of this study was to better define the mechanism/s and signalling pathways that modulate the ESE-1-driven *MMP13* promoter activation in chondrocytes.

Methods: We investigated the contribution of ESE-1 to MMP-13 expression by siRNA knock down (KD) transfection experiments in human primary chondrocytes and real time PCR analysis of MMP-13 expression induced by IL-1 β . The response of the *MMP13* promoter to ESE-1 was analyzed in luciferase reporter assays, after co-transfection of immortalized chondrocytes with human *MMP13* promoter constructs and expression vectors encoding ESE-1, p38, JNK, ERK-1, MKK-6, MKK-7, MEK-1 and MKP-1. ESE-1 binding to the *MMP13* promoter was analyzed by chromatin immunoprecipitation (ChIP) assays. IL-1 β -induced ESE-1 nuclear translocation was addressed by Western blotting analysis of cytoplasmic and nuclear fractions of human immortalized chondrocytes.

Results: Real time PCR analysis revealed a significant reduction of IL-1 β -induced MMP-13 mRNA levels associated with ESE-1 KD in human primary chondrocytes. Luciferase reporter assays showed that MEK1/ERK1 overexpression enhanced ESE1-driven activities of the -1528/+27 and -267/+27 *MMP13* promoter constructs, whereas MKK6/p38 or MKK7/JNK overexpression did not affect ESE-1 transactivation of MMP-13. Overexpression of MKP-1 reduced both the ESE-1 activation of MMP13 and also the MEK1/ERK1 enhancement of ESE-1-driven MMP13 activation. Accordingly, pre-treatment with the MEK1/2 inhibitor, U0126, decreased the *MMP13* promoter activity induced by ESE-1 overexpression. Finally, Western blotting analysis revealed that IL-1 β stimulation induced ESE-1 nuclear translocation, which correlated with increased ESE-1 binding to the endogenous *MMP13* promoter, as addressed by ChIP assays.

Conclusions: The role of MMP-13 as a central factor for OA progression has been highlighted by recent studies in *Mmp13* knockout mice. We previously reported that ESE-1 is a key factor in controlling *MMP13* transcription. ESE-1 belongs to the ETS family of transcription factors, which are classic MAPK effectors in different tissues and cell types. Here, we show that ESE-1 participates in the IL-1 β -induced MMP-13 expression and that IL-1 β induces ESE-1 nuclear translocation and binding to the *MMP13* promoter. In addition, we show that the ESE1-driven transactivation of *MMP13* is enhanced by MEK1/ERK1. In chondrocytes, ERK1/2 phosphorylation is increased *in vitro* in response to IL-1 β and *in vivo* in OA cartilage, and MEK/ERK activation has been reported to participate in the cytokine-induced MMP-13 expression. Therefore, ESE-1 could be one of the effectors of the MEK/ERK pathway in controlling MMP-13 expression in chondrocytes, and the relative contributions of the MEK/ERK/ESE1 axis in OA with regard to MMP-13 expression and activity will merit further investigation.

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TEMPORAL EXPRESSION AND TISSUE DISTRIBUTION OF INTERLEUKIN-1 β IN TWO STRAINS OF GUINEA PIGS WITH VARYING DEGREES OF SPONTANEOUS OSTEOARTHRITIS

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Purpose: Interleukin-1 β (IL-1 β) has been cited as a major cytokine involved in OA-related joint degeneration but definitive characterization of its role *in vivo* remains elusive. The objective of this study was to determine the temporal expression and tissue distribution of IL-1 β in whole knee joints harvested from OA-prone and resistant guinea pigs via immunohistochemistry (IHC). Two main hypotheses were tested: 1) differences in IL-1 β expression would be detected in joint tissue of OA-prone animals

during the course of disease progression; and 2) IL-1 β expression patterns in OA-prone animals would differ from that of OA-resistant animals.

Methods: OA-prone Hartley (N=24) and OA-resistant Strain 13 (N=24) guinea pigs were collected at 60, 120, 180, 240, 360, and 480 days of age (N=4 per strain per time point). IHC was performed on paraffin-embedded sections of whole knee joints and the distribution of IL-1 β expression in all tissue was both descriptively described and scored. OA was graded on toluidine blue stained coronal sections using Mankin criteria with modifications specific for the guinea pig. Data was reported as median and range, and statistical analysis was performed using the Kruskal-Wallis one-way analysis of variance by ranks followed by Dunn's post test.

Results: Median OA scores for both strains increased from 60 to 480 days of age, and a statistically higher score ($p \leq 0.01$) was found in Hartley animals by 480 days of age. The tissue distribution and intensity of IL-1 β immunostaining was consistent within the knee joint organs for each group (strain and age) examined, allowing identification of differing expression patterns in cartilage, synovium, menisci, and subchondral bone both within and between guinea pig models over time. The most striking finding was persistent IL-1 β expression in all aforementioned tissues in Hartley guinea pigs at 120 and 180 days of age, while Strain 13 animals demonstrated a significant ($p \leq 0.05$) and marked reduction in positive cells and/or matrix at these same time points. Further, when present, the distribution of positive protein detection often varied within tissue type for each guinea pig strain. For example, descriptive regional differences in weight-bearing cartilage were discerned between guinea pig strains at 360 and 480 days of age. Immunostaining in the cartilage of Hartley guinea pigs at these time points was restricted to midline and medial or lateral regions, while positive cells and/or matrix of Strain 13 animals were present only in central regions.

Conclusions: This study provides evidence that IL-1 β continues to be an important biomarker relevant to the development and progression of OA. Although these findings do not directly explicate a cause-and-effect relationship between IL-1 β and OA, data is provided as to a window of time when targeted cytokine reduction and/or blockade may prove effective in elucidating such a correlation and identifying its mechanistic components. In this vein, *in vitro* and *in vivo* investigations are underway to determine the effects that reduction of IL-1 β via RNA interference may have on other key mediators implicated in OA. Alternately, should IL-1 β , itself, prove to represent only a secondary response to a more primary insult propagating OA pathogenesis, groundwork has been laid to hone in on the offending pathway.

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THE COMBINATION OF IL-4 AND IL-10 PROTECTS AGAINST BLOOD-INDUCED CARTILAGE DAMAGE

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Purpose: Exposure of cartilage to blood can occur after joint trauma, during or after major joint surgery, or in hemophilia patients. This ultimately leads to joint damage, having both the inflammatory characteristics of rheumatoid arthritis and the degenerative characteristics of osteoarthritis. It was shown that *in vitro* exposure of cartilage to 50% v/v blood for 4 days, the supposed natural evacuation time of blood from a joint, leads to 98% inhibition of cartilage matrix synthesis. Even after a recovery period of 12 days (in the absence of blood) cartilage matrix synthesis is still inhibited by 78%. We have previously demonstrated that interleukin (IL)-10 limits *in vitro* blood-induced cartilage damage. In addition, IL-4 has been suggested to have cartilage protective properties. Hence our aim was to study whether IL-4 can support IL-10 in the prevention of blood-induced cartilage damage.

Methods: Human full thickness articular cartilage explants were cultured for 4 days in the presence or absence of 50% v/v homologous blood. IL-4 was added in concentrations of 0, 1, 3, 10, 30, or 100 ng/ml during blood exposure. A combination of IL-4 and IL-10 was added in a concentration of 100 ng/ml. After 4 days the medium was refreshed and cartilage was cultured for an additional 12 days in the absence of additives to determine long-term effects of short-term blood-exposure and treatment. Cartilage matrix turnover, in terms of proteoglycan synthesis, -release, and -content, was determined at day 16.

Results: Cartilage cultured in the presence of blood showed a decrease of proteoglycan synthesis rate of 70%, an increase of proteoglycan release of 100%, and a decrease of proteoglycan content of 15% after 16 days of culture (all $p < 0.05$). This blood-induced damage of the cartilage matrix was limited by IL-4 in a clear dose-dependent way. Addition of 100 ng/ml IL-4 during blood-exposure reduced the proteoglycan synthesis rate with only 45%, and decreased the proteoglycan release with 30% compared to control (all $p < 0.05$). Moreover, proteoglycan content was normalized. The combination of IL-4 and IL-10 was clearly more protective against damage caused by blood. This was especially evident for the proteoglycan synthesis which was completely normalized. Furthermore, treatment with a combination of the two cytokines was significantly better than the effect of IL-4 and IL-10 alone ($p < 0.05$).

Conclusions: Besides IL-10, as shown previously, also IL-4 protects against blood-induced cartilage damage. The combination of these two cytokines is clearly the most protective. In addition to the direct effects on cartilage, both cytokines are synergistic in inhibition of inflammation. As such, this justifies further evaluation of the combination of IL-4 and IL-10 in prevention and treatment of blood-induced joint damage.

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BRADYKININ, THROUGH B₂ RECEPTORS, ACTIVATES THE RELEASE OF THE CYTOKINE INTERLEUKIN 6, THE CHEMOKINE INTERLEUKIN 8, AND THE METALLOPROTEINASE 3 IN HUMAN KNEE CHONDROCYTES

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Purpose: Bradykinin (BK, H-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) is a proinflammatory and algogenic peptide: it releases inflammatory mediators and sensitizes sensory afferents through the activation of B₂ receptors expressed on the membrane of several cell types, including synoviocytes and chondrocytes. Aim of the current investigation was to investigate if BK and B₂ receptor can participate into mechanisms which are involved into osteoarthritis (OA) degenerative events, and to evaluate the possibility to prevent them through B₂ receptor blockade, using the highly selective and potent B₂ receptor antagonist MEN16132 (m.w. 873.16).

Methods: Human chondrocytes (Lonza, CC-2550) were cultured in F12/DMEM 1:1 added with CGM Singlequots (Lonza, CC-4409) and Gln 2mM and used up to fifth passage. Experiments were performed with cells at confluence plated onto 24-well plates. Cells were incubated at the indicated concentration of BK in F12 medium supplemented with foetal bovine serum (1%), penicillin (50 µg/ml), streptomycin (50 µg/ml), amphotericin B (0.75 µg/ml), Gln (2 mM), and captopril (1 µM). At the end of the experiments, supernatants were collected and stored at -80°C and used for the dosage of interleukin 6 (IL-6), interleukin 8 (IL-8), and metalloproteinase 3 (MMP3). IL-6, IL-8, and MMP3 contents in the supernatant were assayed by commercially available enzyme immunoassay kits (Promokine PK-EL-61606, PK-EL-61806, and Biosource KAC1541). Data are expressed as mean \pm s.e.m. or 95% confidence limits in parentheses of 3 to 5 experiments, each in triplicate.

Results: Time-course experiments (2 - 96 h) indicated that BK (1 µM) induced a release of IL-6, IL-8, and MMP3 which increased over the time, stably peaked after 24 h of incubation, and remained constant up to 96 h. The maximal production of IL-6, IL-8 and MMP3 induced by BK (24 h incubation) was 557 \pm 39 pg/ml, 1267 \pm 169 pg/ml, and 5.52 \pm 0.73 ng/ml, respectively and was resembling that induced by the pleiotropic cytokine TNF α (0.1 ng/ml) (817 \pm 350 pg/ml, 1778 \pm 198 pg/ml, and 1.96 \pm 0.2 ng/ml, respectively). Concentration-response curves to BK (0.1 nM - 1 µM, 24 h) indicated EC₅₀ values of 10 nM (5.4-18.6) in inducing an augmented release of IL-6, 9.7 nM (3.7-32.5) for the release of IL-8, and 9.1 nM (1.1-7.6) for MMP3 secretion. This effect provoked by BK (100 nM, submaximal concentration) were concentration-dependently prevented by the pretreatment of HCCK (30 min) with the selective B₂ receptor antagonist MEN16132, and IC₅₀ values were 1.7 nM (1.0-2.8) to inhibit IL-6 production, 2.2 nM (0.7-7.1) for IL-8, and 0.7 nM (0.3-1.4) for inhibition of MMP3 secretion.

Conclusions: These findings disclose novel actions of BK that imply its possible involvement in joint degenerative diseases, and indicate B₂ receptor blockade as a potential therapy in OA pathology.

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CARTILAGE THICKENING IN EARLY RADIOGRAPHIC KNEE OSTEOARTHRITIS - FACT OR ARTIFACT?

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Purpose: In animal studies of osteoarthritis (OA), increases in cartilage thickness have been observed at the early phases, due either to cartilage hypertrophy or edema. Evidence for cartilage thickening in human OA, however, remains enigmatic. Cross sectional analyses and one longitudinal study in human with MRI have indicated that medial osteophytes, in the absence of medial joint space narrowing (JSN), may be associated with cartilage thickening in the medial femorotibial compartment in human OA, particularly in the external aspect of the medial femoral condyle (ecMF). However, comparisons may have been confounded by differences in age, BMI and other factors.

To eliminate confounding by between-person differences and to enhance sensitivity, we performed a within-person between-knee analysis of cartilage thickness in knees with unilateral osteophytes and without JSN. The primary endpoint was the between-knee cartilage thickness differences in ecMF (in subjects with unilateral medial osteophytes), the secondary endpoint was thickness differences in the external lateral femur (in those with unilateral lateral osteophytes), and the exploratory endpoints were thickness differences in all other femorotibial subregions.

Methods: 83 OA Initiative participants were selected from 4800 cases (public use data sets 0.E.1 [imaging] and 0.2.2 [clinical]), who displayed definite osteophytes and no JSN in one knee and no signs of radiographic OA in the other knee, according to the site readings. The radiographs were reviewed by a central reader (F.R.) and 61 participants were included. Cartilage thickness was measured in four femorotibial cartilage plates (medial/lateral, femur/tibia) and in 16 femorotibial subregions, using sagittal DESSw MR images and dedicated software (Chondrometrics). Within-person between-knee differences were calculated with a paired t-test and general linear models. No adjustment for multiple comparisons was performed.

Results: Of the 61 participants (age 60.8 \pm 9.6 yrs; 29m/32f; BMI 27.8 \pm 4.7) 48% displayed medial, 36% lateral, and 16% bicompartimental (mostly tibial) osteophytes. Knees with osteophytes had thicker cartilage than contralateral knees in the external medial (ecMF: +5.5%, $p=0.02$) and external lateral femur (ecLF: +4.1%, $p=0.01$), but in no other subregions. Knees with only medial osteophytes displayed greater thickening in ecMF (+7.8%) than ecLF (-0.1%), those with only lateral osteophytes greater thickening in ecLF (+5.0%) than in ecMF (+1.7%), and those with bicompartimental osteophytes similar thickening in both subregions (+17%/+15%, respectively). There was no significant effect of age, sex, or BMI on between-knee differences.

Conclusions: Knees with early radiographic OA (definite osteophytes, no JSN) displayed thicker cartilage than the contralateral knees without radiographic OA. The differences were specific to external medial femoral and external lateral femoral subregions and appeared to be locally mediated by (tibial) osteophytes in the same compartment.

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SUPEROXIDE DISMUTASE 2 DOWNREGULATION AND MITOCHONDRIA RESPIRATION IN OSTEOARTHRITIS

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Purpose: Oxidative phosphorylation takes place at the mitochondrial respiratory chain and is the major source for the production of ATP. A by-product of this respiration are reactive oxygen species (ROS). ROS are involved in signalling processes but when at high levels contribute to oxidative damage. The major ROS include superoxide (O₂^{•-}) and hydrogen peroxide (H₂O₂). The mRNA and protein levels of the major inhibitor of superoxides in the mitochondria, superoxide dismutase 2 (SOD2), have been shown to be downregulated in osteoarthritic compared to healthy joint cartilage.

This study characterises the effects of reduced levels of SOD2 on chondro-